PROCESS FOR IDENTIFYING SUBSTANCES WHICH MODULATE THE ACTIVITY OF HYPERPOLARIZATION-ACTIVATED CATION CHANNELS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to the field of biological cell-to-cell communication and electrochemical signalling between biological cells. In particular, the present invention provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, and the use of this process.

Description of the Relevant Art

Some genes of murine and human hyperpolarization-activated cation channels are already known. Examples include muHCN2(muHAC1) (Ludwig et al. (1998)), huHCN4 (Ludwig et al. (1999)), huHCN2 (Vaccari, T. et al. (1999) Biochim. Biophys. Acta 1446(3): 419-425), and those disclosed in WO 99/32615 and WO 99/42574. See, also, Tables 1-6 herein.

Ludwig et al. (1998) have shown that muHCN2 can be transfected transiently in HEK293 cells, and that the corresponding channel in the transfected cells can be examined easily by electrophysiological methods (patch-clamp studies). The electrophysiological properties of the cloned channel correspond to the I_f or I_h current described in pacemaker cells, which had hitherto not been known on a molecular level (Ludwig et al. (1998), Biel et al. (1999)). The channel activates when the holding potential is changed toward hyperpolarization (potential at about B100 to B160 mV). However, the patch-clamp technique cannot be automated and is not suitable for high-throughput screening (HTS).

Using suitable dyes, ion currents can be measured in an FLIPR (fluorescence imaging plate reader; Molecular Devices, Sunnyvale CA, USA). Influx or efflux of ions leads to

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changes in the membrane potential, which can be measured in high-throughput screening in an FLIPR using suitable fluorescent dyes. However, in contrast to the patch-clamp method, it is not possible to generate voltage changes in the FLIPR. Voltage changes are, however, an essential prerequisite for the activation of hyperpolarization-activated cation channels.

SUMMARY OF THE INVENTION

For the examination of the largest possible number of substances, we have developed a process that permits, among other things, high-throughput screening (HTS) for modulators of a hyperpolarization-activated cation channel.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: The figure shows the results of practicing an assay according to the invention. The change of the measured fluorescence over time is presented as the mean of, in each case, 24 wells. A and B were obtained with cells which, during dyeing, contained 10 µM forskolin in the dyeing medium. C and D are curves of cells that did not get any forskolin.

Fig. 2: The figure shows the effect of zatebradine on the change in fluorescence over time using an assay according to the invention. Curve A shows the uninhibited change over time (only 50 mM NaCl). Curve E shows the change over time, inhibited by CsCl (50 mM NaCl + 8 mM CsCl). Curves B-D show the effect of zatebradine: B: 12.5 µM, C: 25 µM and D: 50 µM.

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DETAILED DESCRIPTION OF THE INVENTION

Abbreviations used herein are listed in Table 7 below.

The present invention provides a way to hyperpolarize cells that express a hyperpolarization-activated cation channel (i.e. to activate the hyperpolarization-activated cation channel) and to maintain this hyperpolarization of the cell, for example, until a measurement of membrane potential can be taken. Under physiological conditions, a hyperpolarization of the cell that is sufficient to activate a hyperpolarization-activated cation channel is reversed by the activity of that channel. Only when hyperpolarization can be maintained is it possible to measure, for example in an FLIPR, the depolarization of the cell caused under suitable conditions by a substance that modulates the activity of the hyperpolarization-activated cation channel.

Generally speaking, the present invention provides a process for examining hyperpolarization-activated cation channels. In the process, cells that express the hyperpolarization-activated cation channels are hyperpolarized B i.e. the hyperpolarization-activated cation channel is activated B and this hyperpolarization of the cells, which is reversed under physiological conditions by the activity of the hyperpolarization-activated cation channel, is maintained. By exclusion of extracellular sodium ions, the activated channel is unable to transport sodium ions into the cells, i.e. to depolarize the cells. If, simultaneously or even prior to the addition of the sodium ions, substances are added that modulate the activity of the hyperpolarization-activated cation channel, the depolarization is affected. For example, compared to when only sodium ions are added, depolarization is increased in the case of HCN activators (for example forskolin) and reduced in the case of HCN inhibitors (for example zatebradine = 3-[3-[[2-(3,4-dimethoxyphenyl)ethyl]methylamino]propyl]-1,3,4,5-tetrahydro-7,8-dimethoxy-2H-3-benzazepin-2-one; Reiffen et al. (1990)).

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By measuring the depolarization of the cells or the changes of their membrane potential, it is possible to identify substances that modulate the activity of the hyperpolarization-activated cation channel.

In one aspect, the invention generally provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein

a) cells which express a hyperpolarization-activated cation channel are used;

b) the cells are hyperpolarized in the presence of a potential-sensitive fluorescent dye using an isoosmolar sodium-ion-free buffer; and

c) the change in the membrane potential of the cells following simultaneous addition of sodium ions and the substance to be examined is detected and recorded.

Thus, in embodiments, the invention provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein the process comprises

a) providing, in a suitable container, cells that express a hyperpolarization-activated cation channel;

b) hyperpolarizing the cells in the presence of a potential-sensitive fluorescent dye and an isoosmolar sodium-ion-free buffer;

c) optionally, determining the membrane potential of the cells;

d) simultaneously adding sodium ions and a sample containing at least one substance to be tested for its ability to modulate the activity of the cation channel;

e) determining the membrane potential of the cells;

f) determining whether the membrane potential changed upon simultaneous addition of sodium ions and the substance(s); and

g) optionally, recording the change in membrane potential,

wherein a change in membrane potential indicates the presence of at least one substance in the sample that modulates the activity of the cation channel.

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A suitable container is any container, vessel, receptacle, etc. that can be used to hold the reagents and samples to be used in the assay. Suitable containers are disclosed in, or identifiable from, literature provided by manufacturers of equipment designed to determine membrane potentials. Such equipment is publicly available and well known to those of skill in the art.

In embodiments where step "c)" is not performed, a parallel assay, using the same strain of cells at the same concentration in the same assay composition, can be run to determine the membrane potential of the cells in the absence of the sample suspected of containing at least one substance that can modulate the activity of a cation channel.

In embodiments, the assay is a high-throughput assay.

In another aspect, the invention generally provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein

a) cells which express a hyperpolarization-activated cation channel are used;

b) the cells are hyperpolarized in the presence of a potential-sensitive fluorescent dye using an isoosmolar sodium-ion-free buffer;

c) the cells are incubated with a substance to be examined; and

d) the change in the membrane potential of the cells after addition of sodium ions is detected and recorded.

Thus, in embodiments, the invention provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein the process comprises

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a) providing, in a suitable container, cells that express a hyperpolarization-activated cation channel;

b) hyperpolarizing the cells in the presence of a potential-sensitive fluorescent dye and an isoosmolar sodium-ion-free buffer;

c) optionally, determining the membrane potential of the cells;

d) incubating the cells with a sample containing at least one substance to be tested for its ability to modulate the activity of the cation channel;

e) optionally, determining the membrane potential of the cells;

f) optionally, determining whether the membrane potential changed upon addition of the substance(s) to be tested;

g) adding sodium ions;

h) determining the membrane potential of the cells;

i) determining whether the membrane potential changed upon addition of the sodium ions; and

j) optionally, recording the change in membrane potential,

wherein a change in membrane potential between the time before the sodium ions are added and after the sodium ions are added indicates the presence of at least one substance in the sample that modulates the activity of the cation channel.

Extracellular potassium ions can be included in the assay. In certain situations, these ions can improve the function of the hyperpolarization-activation cation channels. For example, they might be included when HCN (HAC) channels are being used in the process. Thus, in embodiments of the present invention, the isoosmolar sodium-ion-free buffer comprises potassium ions (K^+). In embodiments, the buffer comprises potassium ions in the form of potassium chloride. In embodiments, the buffer comprises potassium ions at a concentration of at least about 0.5 mM K^+. In embodiments, the buffer comprises potassium ions at a concentration of at least about 0.8 mM K^+. In embodiments, the buffer

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comprises potassium ions at a concentration of about 2 mM. In embodiments, the buffer comprises potassium ions at a concentration of about 5 mM.

In embodiments, the isoosmolar sodium-ion-free buffer comprises at least one cation that is not able to cross the membrane in amounts that correspond to the normal extracellular sodium ion concentration. For example, the buffer can comprise choline, for example in the form of choline chloride, or NMDG (N-methyl-D-glucamine). In embodiments, the isoosmolar sodium-ion-free buffer comprises both potassium ions and at least one cation that is not able to cross the membrane in amounts that correspond to the normal extracellular sodium ion concentration.

In embodiments, the isoosmolar sodium-ion-free buffer comprises a potential-sensitive dye, for example a potential-sensitive fluorescent dye. Included among these are oxonol derivatives, such as 3-bis-barbituric acid oxonol. Thus, in embodiments, the isoosmolar sodium-ion-free buffer comprises potassium ions, at least one cation that is not able to cross the membrane in amounts that correspond to the normal extracellular sodium ion concentration, and a potential-sensitive dye.

In embodiments, the buffer comprises potential-sensitive fluorescent dyes that are suitable for examining the membrane potential of nonexcitable cells. Examples of such dyes include, but are not limited to, potential-sensitive slow-response dyes. Non-limiting examples of such potential-sensitive slow-response dyes include bis-(1,3-dibutylbarbituric acid)trimethine oxonol [$DiBac_4(3)$], bis-(1,3-diethylthiobarbituric acid)trimethine oxonol [$DiSBac_2(3)$] or bis-(1,3-dibutylbarbituric acid)pentamethine oxonol [$DiBac_4(5)$]. Other known and suitable potential-sensitive dyes include, but are not limited to, fast-response dyes (for example, of the styrylpyridinium type), which are used in certain embodiments in conjunction with excitable cells, such as neurons, cardiac cells, etc. These potential-sensitive dyes react in the millisecond range and are not particularly sensitive (2-10%

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fluorescence change per 100 mV potential change). Other suitable dyes include slow-response dyes of the carbocyanine type. Non-limiting examples of these slow-response dyes include diOC5(3)-3,3'-dipentyloxacarbocyanine iodide, diOC6(3)-3,3'-dihexyloxacarbocyanine iodide, etc.), JC-1 (5,5',6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolecarbocyanine iodide), and rhodamine 123. In embodiments, these slow-response potential-sensitive dyes are used in studies of the membrane potential of mitochondria.

One embodiment of the invention relates to the use of the fluorescent dye from the FLIPR Membrane Potential Assay Kit (Molecular Devices, Sunnyvale, CA, USA). The fluorescence of this dye can be measured using a standard emission filter, which is transparent between about 510 and about 580 nm. In embodiments, fluorescence of this dye is measured using a filter that is transparent above about 550 nm. The manufacturer of this dye and kit disclose a number of advantages of their product, over, for example, assays based on $DiBac_4(3)$, and these advantages can be applicable to the present invention.

Some of these advantages include:

1) the measurement of membrane potentials with the kit is not temperature sensitive, in contrast to $DiBac_4(3)$, where the temperature has to be equilibrated prior to the actual measurement in the FLIPR;

2) the volume added in the FLIPR can be greater than that in the case of $DiBac_4(3)$, where usually all substances have to be added in a 10-fold concentrated form;

3) the measurements can be carried out much more rapidly, since the kit requires a much shorter time to reach the steady state than $DiBac_4(3)$, which usually requires between 10 and 30 minutes;

4) for many measurement protocols, a washing step prior to the addition of the dye is no longer required; and

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5) the dye does not have to be present in each solution.

In embodiments of the present invention, the first two advantages are relied upon because these two advantages can be applied to assays of hyperpolarization-activated cation channels. The first two advantages can also be applied to embodiments of the invention that are directed to high-throughput screening, since screening of a large number of samples at once can be complicated and/or time consuming. For example, in embodiments where FLIPR II, which allows the measurement in 384-well plates and which is preferably employed for high-throughput screening thermostating, is used, these first two advantages can reduce the complications and time necessary to perform the assay process. In the case of poorly soluble substances, it is furthermore an advantage if they can be added to the cells in five-, three-, or even two-fold concentrated form instead of 10-fold concentrated form, as is typical with $DiBac_4(3)$.

In the processes of the present invention, cells having an elevated intracellular cAMP concentration can be used. Elevated intracellular cAMP concentrations can be achieved, for example, by adding cAMP derivatives that are able to cross the membrane. Non-limiting examples of such derivatives include dibutyryl-cAMP and 8-bromo-cAMP. As a further non-limiting example, the intracellular cAMP concentration can be increased by the addition of an adenylate cyclase activator, for example forskolin. When forskolin is used, successful results can be obtained when it is supplied in concentrations of less than about 100 µM. For example, forskolin can be used at a concentration of between about 1 µM and about 100 µM. It can also be used at concentrations less than about 1 µM. In embodiments, it is used at a concentration of about 10 µM. In principle, it is also possible to use all substances or ligands that activate adenylate cyclase by signal transduction in the cell line employed (for example ligands for b-adrenergic receptors, such as adrenalin, isoproterenol, noradrenalin, etc., if the cell has endogenous b-adrenergic receptors).

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To depolarize the membrane potential, Na^+ (which can be supplied in the form of NaCl, for example) is added in the FLIPR to the cells which have hyperpolarized by the sodium-ion-free buffer. In embodiments, the Na^+ is added to achieve a final Na^+ concentration of about 20-100 mM. In embodiments, it is added to achieve a final Na^+ concentration of about 50 mM. In embodiments where the FLIPR Membrane Potential Assay Kit (Molecular Devices, Sunnyvale, CA, USA) is used, the final Na^+ concentration can be about 20-100 mM. For example, it can be about 40-80 mM.

In embodiments, the invention relates to processes in which the hyperpolarization-activatable cation channel is an HCN1, HCN2, HCN3, HCN4 channel (where HAC1=HCN2, HAC2=HCN1, HAC3=HCN3 and HAC4=HCN4) or a KAT1 (=AKT) channel (hyperpolarization-activated potassium channel from Arabidopsis thaliana); a heteromultimer of these channels (i.e. a channel which is composed of subunits of different hyperpolarization-activated cation channels); or a chimeric hyperpolarization-activated cation channel (i.e. a synthetic channel in which individual subunits are composed of parts of different channels or hyperpolarization-activated cation channels). The hyperpolarization-activated cation channel is preferably a human hyperpolarization-activated cation channel, for example huHCN2, (SEQ ID NO. 1, SEQ ID NO. 2) or huHCN4 (SEQ ID NO. 3, SEQ ID NO. 4), or a murine hyperpolarization-activated cation channel muHCN2 (SEQ ID NO. 5, SEQ ID NO. 6). See Tables 1-6. On the amino acid level, the identity between muHCN2 and huHCN2 is 94.8%. In principle, the process is suitable for all cation channels which are activated by hyperpolarization. For example, it is suitable for HCN1-4 (or HAC1-4; see Biel et al. (1999)).

The cells can be any eukaryotic cells. For example, the cells can be mammalian cells, such as CHO or HEK293 cells. In embodiments, CHO cells or another cell line having comparably few endogenous potassium channels are used, since endogenous potassium channels might interfere with the measurement, for example, in the FLIPR. In other

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embodiments cells whose endogenous potassium channels are not functionally expressed (for example the corresponding knock-out cells) are used.

The cells can, but do not necessarily, contain nucleic acids (i.e., RNA, DNA, PNA) that codes for the hyperpolarization-activated cation channel. In embodiments, the cells contain DNA. In embodiments, the cells contain RNA. In embodiments, the cells contain a cDNA of a hyperpolarization-activated cation channel in a suitable plasmid. Such cells can be prepared by transfecting the original cell line with a plasmid that contains the cDNA of a hyperpolarization-activated cation channel. Other techniques can be used as well. Techniques for introducing heterologous nucleic acids into cells are well known and widely practiced by those of skill in the art, and thus need not be detailed here.

In the case of the hyperpolarization-activated cation channels, it is an object of the invention to detect, and optionally, record changes in the membrane potential of the cells, where the changes are the result of the activation or the inhibition of these channels. Detection can utilize bis-barbituric acid oxonols. Three bis-barbituric acid oxonols (see, for example, "Handbook of Fluorescent Probes and Research Chemicals", 6th edition, Molecular Probes, Eugene OR, USA), which are mainly referred to as DiBac dyes, form a family of potential-sensitive dyes having excitation maxima at 490 nm ($DiBac_4(3)$), 530 nm ($DiSBac_2(3)$), and 590 nm ($DiBac_4(5)$). The dyes get into depolarized cells by binding to intracellular proteins or membranes, leading to increased fluorescence and a red shift. Hyperpolarization results in the expulsion of the anionic dyes and thus in a decrease in fluorescence. This decrease in fluorescence can be measured, for example, with the measuring device FLIPR. Accordingly, one embodiment of the invention relates to the measurement of the membrane potential in a Fluorescent Imaging Plate Reader (FLIPR).

The FLIPR (for: Fluorescent Imaging Plate Reader; Manufacturer: Molecular Devices, Sunnyvale, CA, USA) is a measuring device that allows the simultaneous measurement

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of changes of the fluorescence intensity in all wells of a microtiter plate. The dyes used are excited at about 488 nm using an argon laser, which is integrated into the system. The standard emission filter of the system is transparent in the range from 510 B 580 nm. The emitted fluorescence is registered using a CCD camera, and the system permits the simultaneous recording, within an interval of about one second, of the fluorescence in all wells of a 96-well or 384-well microtiter plate. Using a built-in pipettor, it is even possible to determine the fluorescence during the addition of the substance, which can be beneficial, for example, in the case of rapid processes. By means of special optics, the fluorescence can be registered in a layer of only about 50 mm, but not in the entire well. This can be beneficial for background reduction in all measurements where the fluorescent dye is also present extracellularly. Such a situation can exist, for example, in the measurement of changes in membrane potential using DiBac dyes. Standard applications of the system are measurements of the intracellular calcium concentration or the membrane potential of cells. Among the dyes mentioned above, $DiBac_4(3)$, which, owing to its excitation maximum, is most suitable for the argon laser in the FLIPR, has the highest sensitivity for voltage differences.

Since the $DiBac_4(3)$ takes some time to come to equilibrium, the measurement can be taken after a certain incubation time. In embodiments, the incubation temperature is at or about the optimal temperature for growth and metabolism of the biological cells being used in the assay. For example, the incubation temperature can be at or about 37°C. Incubation time can be varied to achieve complete or uniform sample temperature. In embodiments, the sample can be incubated for at least about 10 minutes. In embodiments, the sample is incubated for about or precisely 30 minutes.

Although results can be obtained at any time desired, in order to obtain as reliable of a result as possible or practical, the results should be determined and, optionally, recorded as quickly as possible after each incubation step. This is because cooling of the dye

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solution might affect the result of the measurement. Thus, prior to any measurement, the composition to be measured can be incubated at a chosen temperature for a period of time that is sufficient to equilibrate the temperature of the composition at a desired level. For example, the composition can be incubated for at least about one minute, or at least about two, three, for, five, or even more minutes. Included are incubation periods prior to initial measurements (e.g., to determine base-line levels of activity or membrane potential). As with the other incubation periods, this pre-incubation phase can be carried out to compensate for temperature variations on the microtiter plate.

In embodiments where FLIPR is used, the measurement is typically carried out using the temperature parameters preset by the FLIPR manufacturer for the measurement of membrane potentials (about 37°C). However, this is a guideline, and those practicing the invention can alter the temperature to achieve maximal results. Such temperature modifications are well within the skill of those in the art, and do not represent undue experimentation. In embodiments, the parameters preset by the FLIPR manufacturer are followed essentially precisely.

Although variations in volume can be accounted for, in the FLIPR, in embodiments of the present invention, the volume of the reaction solution in which the process is carried out is changed as little as possible. In embodiments where $DiBac_4(3)$ is used, the $DiBac_4(3)$ signal is most reproducible if only relatively small volume changes take place in the FLIPR; thus, the volume is typically maintained throughout, to the extent possible and practicable. Accordingly, in these embodiments, the substances to be tested are added as concentrated solutions. In embodiments, they are added at a concentration of at least about 2-fold. For example, they can be added in about a five-fold, ten-fold, or even greater concentrated form to the $DiBac_4(3)$-dyed cells.

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Since the fluorescence measurement with the FLIPR Membrane Potential Assay Kit is not temperature-sensitive, it can be carried out simply at room temperature. This can be advantageous, for example, in embodiments that utilize the FLIPR II, which allows measurements with 384-well microtiter plates.

In embodiments, the HCN channels are activated by hyperpolarization (for example HCN2 at B100 mV to about 50%) and cause a depolarization of the cells. By increasing the intracellular cAMP concentration (for example with dibutyryl-cAMP or with forskolin), the value of the half-maximal activation can be shifted by about 10 mV to more positive potentials (Ludwig et al., 1998).

Electrophysiologically, HCN channels can be studied easily on stably transfected cells using the patch-clamp method, as voltage changes can be brought about easily. In contrast, in the FLIPR, it is not possible to induce voltage changes, and exactly because of the HCN activity, a hyperpolarization of the cells would only be transient. It has not been possible to achieve hyperpolarization of the transfected cells by adding an HCN2 inhibitor (zatebradine), since the resting membrane potential of the transfected cells is much too far removed from the potentials at which HCN2 is activated.

On the one hand, hyperpolarization is required for HCN activation. However, on the other hand, under physiological conditions, an activated HCN leads immediately to depolarization. Accordingly, in the present invention, conditions are provided under which the HCN channels can be activated by hyperpolarization, but where depolarization by the activated HCN channel is initially impossible. To this end, the cells, for example cells seeded in microtiter plates, are washed in an isoosmolar buffer in which NaCl has been replaced by another chloride salt, such as choline chloride. In embodiments, the wash buffer also contains at least some KCl, since extracellular K^+ can improve HCN activation (Biel et al. 1999). In embodiments, the wash buffer contains at least 1mM KCl. In

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embodiments, the wash buffer contains about 5 mM KCl. The wash buffer, which serves to effect hyperpolarization of the cation channels and thus the HCN cells, can also contain 5 µM $DiBac_4(3)$ for measuring changes in the membrane potential in the FLIPR. By removing the extracellular Na^+, the cells are hyperpolarized, i.e. the cation channel is activated. However, the HCN is not capable of causing depolarization of the cells, since the required concentration gradient of the ions Na^+ or K^+ transported by HCN is missing. Here, an activated HCN could only result in a more pronounced hyperpolarization. This is reflected in the fact that the initial fluorescence measured for HCN cells in the FLIPR at 10 µM forskolin is lower than that without forskolin, whereas there is no difference in nontransfected cells.

In the FLIPR, Na^+ is added to the cells, so that the activated HCN (after a few seconds, in which there are mixing effects) causes, from about 15 seconds after the addition of Na^+, depolarization of the cells, which becomes visible by an increase in fluorescence. The detection of HCN modulators can rely on a difference between cells having an activated HCN channel (e.g., only Na^+ addition) and cells having a blocked HCN channel (e.g., Na^+ + 8 mM CsCl). It has been determined that a greater difference provides a greater reliability in the system. For example, activation of the HCN channel by pre-incubation with 10 µM forskolin increases the difference between the uninhibited 100% value from the inhibited 0% value considerably (see Fig. 1).

One embodiment of the present invention relates to the comparative determination of the change in the membrane potential of at least two cell populations incubated with different concentrations of one of the substances to be examined. In this way, the optimal concentration of the substance(s) can be determined.

Substances that are to be examined for their activity are referred to as substances to be examined or substances to be tested. Substances that are active, i.e. that modulate the

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activity of the hyperpolarization-activated cation channel, can either be inhibitors (they inhibit the channel and reduce depolarization or prevent depolarization altogether) or be activators (they activate the channel and cause a more pronounced or more rapid depolarization) of the hyperpolarization-activated cation channel.

In embodiments, the invention provides a high-throughput screening (HTS) process. In HTS, the process can be used for identifying inhibitors and/or activators of a hyperpolarization-activated cation channel. Substances identified in this manner can be used, for example, as pharmaceutically active compounds. Thus, they can be used as medicaments (medicinal compositions) or as active ingredients of medicaments.

Accordingly, the invention also provides a process that comprises the formulation of an identified substance in a pharmaceutically acceptable form. In this aspect of the invention, the methods described above can be linked to formulation of an identified substance in a pharmaceutically acceptable form. Such forms, and processes for preparing such forms, are well known to, and widely practiced by, those of skill in the art. Therefore, they need not be detailed here. Examples include, but are not limited to, forms that comprise excipients or biologically tolerable carriers.

The invention also provides a process for preparing a medicament. The process comprises the identification of a substance that inhibits or activates the activity of a hyperpolarization-activated cation channel, and mixing the identified substance with a pharmaceutically acceptable excipient. In embodiments, the process for preparing a medicament comprises

a) the identification of a substance which modulates the activity of hyperpolarization-activated cation channels;
b) the preparation of the substance;

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c) the purification of the substance; and

d) the mixing of the substance with a pharmaceutically acceptable excipient.

The invention also provides a kit. In embodiments, the kit is a test kit for determining whether a substance modulates the activity of a hyperpolarization-activated cation channel. In embodiments, the test kit comprises

a) cells that overexpress a hyperpolarization-activated cation channel;

b) an isoosmolar sodium-ion-free buffer for hyperpolarizing the cell; and

c) at least one reagent for detection of hyperpolarization activated cation channels.

The components/reagents can be those described in detail herein with respect to the assays of the invention. The components can be supplied in separate containers within the kit or in combinations within containers within the kit. Where applicable, components and/or reagents can be supplied in stabilized form. The stabilized form can permit the components and/or reagents to be maintained for extended periods of time without significant degradation or loss in activity. For example, the cells can be supplied in a cryogenic state. In addition, the salts (ions) or reagents that will comprise the assay composition can be provided in solid (dry) form, to be reconstituted with water or another appropriate solvent prior to use. Accordingly, the kit can comprise water.

As a measure for the activity of a substance, the change in the membrane potential of the cell is measured, for example, with the aid of a potential-sensitive fluorescent dye. As mentioned above, the dye can be an oxanol derivative, such as 3-bis-barbituric acid oxanol.

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Examples

The invention will now be illustrated in more detail by various examples of embodiments of the invention. The following examples are exemplary only. Thus, the scope of the invention is not limited to the embodiments disclosed in the examples. Abbreviations used in the Examples are listed in Table 7 below.

Example 1: Preparation of transfected cells

The plasmid pcDNA3-muHCN2 contains the murine HCN2 (muHCN2) cDNA (Genbank Accession No. AJ225122) of Pos. 22-2812 (coding sequence: Pos. 36-2627), cloned into the *Eco*RI and *Not*I cleavage sites of pcDNA3, and was obtained from M. Biel, TU Munich (Ludwig et al., 1998). In each case 6 µg of this plasmid DNA were used for transfecting CHO or HEK293 cells. For transfecting CHO cells or HEK cells, the LipofectAmine™ Reagent from Life Technologies (Gaithersburg, MD, USA) was used, in accordance with the instructions of the manufacturer. 24 hours after the transfection, the cells were transferred from culture dishes into 75 cm^2 cell culture bottles. 72 hours after the transfection, the cells were subjected to a selection with 400 µg/ml of the antibiotic G418 (Calbiochem, Bad Soden, Germany). Following a two-week selection, the surviving cells were detached from the bottles using trypsin-EDTA, counted in the cell counter Coulter Counter Z1 and sown into 96-well microtiter plates such that statistically, 1 cell was present per well. The microtiter plates were checked regularly under the microscope, and only cells from wells in which only one colony was growing were cultured further.

From these cells, total RNA was isolated with the aid of the QIAshredder and RNeasy kits from Qiagen (Hilden, Germany). This total RNA was examined by RT-PCR for expression of muHCN2 (Primer 1): 5'-GCCAATACCAGGAGAAG-3' [SEQ ID NO. 7], corresponds to Pos. 1354-1370 and AJ225122, and primer 2: 5'-TGAGTAGAGGCGACAGTAG-3' [SEQ ID NO. 8], corresponds to pos. 1829-1811 in AJ225122; expected RT-PCR band: 476 bp.

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Example 2: Patch-clamp examination of the cells

Using the patch-clamp method, the cells with detectable mRNA expression were examined electrophysiologically, in the whole-cell configuration, for functional expression of muHCN2. This method is described in detail in Hamill et al (1981), which is incorporated herein by reference. The cells were clamped to a holding potential of -40 mV. Starting with this holding potential, the ion channels were activated by a voltage change to B140 mV for a period of one second. The current amplitude was determined the end of this pulse. Among the transfected HEK cells, some were found having currents of about 1 nA; however, owing to interfering endogenous currents, it was not possible to construct an assay for these cells in the FLIPR.

However, in the HEK cells, it was found clearly that a functionally active HCN2 channel was only detectable in cells having strong mRNA expression. In the CHO cells, the correlation between mRNA expression and function was confirmed. In general, the mRNA expression in the HEK cells was about three times better than that in the CHO cells. In the patch-clamp studies, it was possible to demonstrate a weak current in some cells of one of the most strongly expressing CHO cell lines.

Example 3: Preparation of doubly-transfected cells

Since the functional expression appeared to correlate strongly with the mRNA expression, we carried out a second transfection with the muHCN2 cDNA that had earlier been cloned into the *Eco*RI and *Not*I site of the vector pcDNA3.1(+)zeo. After a two-week selection with G418 and Zeocin (Invitrogen, Groningen, NL), individual cell clones were isolated as described in Example 1. Following isolation of the total RNA from these cells, an RT-PCR with the primers mentioned in Example 1 was carried out. Then an RT-PCR was carried

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out with the following primers, comprising a region which contains the 3'-end of the coding sequence of muHAC1 (primer 3: 5'-AGTGGCCTCGACCCACTGGACTCT-3' [SEQ ID NO. 9], corresponds to pos. 2553-2576 in AJ225122, and primer 4: 5'-CCGCCTCCTAAGCTACCTACGTCCC-3' [SEQ ID NO. 10], corresponds to pos. 2725-2701 in AJ225122).

Some of the doubly-transfected cells showed a considerably more pronounced expression both in RT-PCR and in the patch-clamp analysis than the cells which had been transfected only once. Electrophysiologically, currents of up to 11 nA were measured. These cells were used for constructing an FLIPR assay for HCN2.

Example 4: Construction of an FLIPR assay for HCN channels

The cells seeded on the microtiter plates are washed in an isoosmolar buffer in which NaCl has been replaced by choline chloride. However, this wash buffer also contains 5 mM KCl, since extracellular K^+ is important for HCN activation (Biel et al. 1999). This wash buffer, which serves to effect hyperpolarization of the HCN cells, also contains 5 µM $DiBac_4(3)$ for measuring changes in the membrane potential in the FLIPR. By removing the extracellular Na^+, the cells are hyperpolarized, i.e. the HCN is activated. However, the HCN is not capable of causing depolarization of the cells, since the required concentration gradient of the ions Na^+ or K^+ transported by HCN is missing. Here, an activated HCN could only result in a more pronounced hyperpolarization. This is reflected in the fact that the initial fluorescence measured for HCN cells in the FLIPR at 10 µM forskolin is lower than that without forskolin, whereas there is no difference in nontransfected cells.

Since $DiBac_4(3)$ fluorescence may be sensitive to temperature variations, the measurement is, after an incubation at 37°C for 30 minutes, carried out as quickly as

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possible - cooling of the dye solution may affect the measured results. Preferably, the sample is thermostated for five minutes in the FLIPR prior to the start of the measurement.

The substances to be tested are preferably added in 10-fold concentrated form to the cells which had been dyed with $DiBac_4(3)$.

In the FLIPR, Na^+ is added to the cells so that the activated HCN (after a few seconds, in which there are mixing effects) causes, from about 15 seconds after the addition of Na^+, depolarization of the cells, which becomes visible by an increase in fluorescence. An activation of the HCN channel by preincubation with 10 µM forskolin increases the difference between the uninhibited 100% value from the inhibited 0% value considerably (see Fig. 1; discussed further below). By comparison with the control values, it can be detected whether a substance to be tested is an activator (more rapid or more pronounced depolarization) or an inhibitor (slower or inhibited depolarization, see Fig. 2: effect of zatebradine).

Figure 1 shows the change of the measured fluorescence over time, as the mean of in each case 24 wells. To be able to see the change over time between 60 and 290 seconds more clearly, the fluorescence measured in each well (sum of the brightness intensities of a fixed number of pixels, constant in all wells) at the time 60 seconds (corresponds to 40 seconds after the addition of the Na^+) was defined as "0" using the FLIPR software. Curves A and B were obtained with cells which, during dyeing, contained 10 µM forskolin in the dyeing medium. C and D are curves of cells which did not get any forskolin. In A and C, NaCl was added in the FLIPR to a final concentration of 50 mM, whereas B and D additionally received 8 mM CsCl to inhibit the HCN2 channel. Using the FLIPR software, the values of curve A (with forskolin; 50 mM NaCl + 8 mM CsCl) were defined as negative controls. Curves B, C, and D represent changes in fluorescence relative to curve A. Compared to A, curve B (with forskolin; no inhibition with CsCl) shows a clear

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depolarization. Compared to curve A, cells which were not preincubated with forskolin but received, simultaneously with NaCl, 8 mM CsCl (curve C) showed a decrease. This indicates that in this assay, forskolin also has an effect on the membrane potential of the cells which is independent of the HCN2 channel that is expressed. Compared to curve C, curve D (no forskolin, no inhibition with CsCl), also shows considerable depolarization. However, since the distance between curves A and B in the interval from 60 seconds onwards is always greater than the distance between curves C and D, it can be seen that forskolin activates the HCN2 channel that is expressed.

Example 5: Determination of the IC50 of an HCN2 blocker

Using the transfected HCN cells, the effect of various concentrations of the substance zatebradine, which is known as an I_f blocker, were examined (see Fig. 2; discussed further below). The inhibition by zatebradine was calculated from the relative change in fluorescence from the time 60 seconds. For each concentration of the inhibitor, the mean of in each case 6 wells of the microtiter plate was determined. From these values, the IC50 of zatebradine was calculated as 26 µM, a value which corresponds well with the value of 31 µM determined electrophysiologically in the same cells.

Figure 2 shows the effect of zatebradine on the change in fluorescence over time. In this figure, all cells received 10 µM forskolin during dyeing. Curve A shows the uninhibited change over time (only 50 mM NaCl), curve E the change over time, inhibited by CsCl (50 mM NaCl + 8 mM CsCl), curves B-D show the effect of zatebradine: B: 12.5 µM, C: 25 µM and D: 50 µM. Curves A and E are each means of the data from 12 wells, curves B, C and D from 6 wells. The data measured following addition of 8 mM CsCl (curve E) were defined as negative controls, so that curves A-D reflect the relative change compared to curve E. It can be seen clearly that the depolarization, which sets in after addition of

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NaCl, decreases with increasing zatebradine concentration. Following export into Excel, the fluorescence data measured in the FLIPR could be used to calculate the IC_{50} for zatebradine (26 µM).

Example 6: Use of the FLIPR Membrane Assay Kit (Molecular Devices, Sunnyvale, USA):

Cells that were seeded a day earlier are, as before, washed three times with in each case 400 µl of wash buffer per well. However, this time, the volume that remains above the cells after the last washing step is chosen depending on the desired Na^+ and Cs^+ concentrations. The dye, in wash buffer, is added, and the cells are incubated with dye for 30 minutes. The temperature is typically room temperature (about 21-25°C), but can be about 37°C.

In the FLIPR, depolarization is then induced by addition of Na^+ and in some control wells inhibited again by simultaneous addition of Cs^+. Since, in the dye from Molecular Devices, an increase in the ionic strength might lead to changes in fluorescence, it has to be ensured that the ionic strength changes to the same degree in all wells of a microtiter plate. The desired final concentrations of sodium or cesium ions permitting, the osmolarity is not changed. To adjust the desired concentrations of Na^+ and Cs^+, two further buffers which, instead of 140 mM of choline chloride, contain 140 mM NaCl (sodium buffer) and 140 mM CsCl (cesium buffer), respectively, are used in addition to the wash buffer.

For measurements with the FLIPR Membrane Potential Assay Kit Molecular Devices gives the following standard protocol for 96-well microtiter plates (384 wells in brackets):
On the day before the measurement, the cells are seeded in 100 ml (25 ml) of medium. Following addition of 100 µl (25 µl) of dye and 30 minutes of incubation at room

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temperature or at 37°C, 50 µl (25 µl) of the substance to be tested, in a suitable buffer, are added in the FLIPR.

Using the volumes stated by Molecular Devices, it is possible, without changing the ionic strength, to achieve a maximum concentration of 28 mM for Na^+ + Cs^+ in 96-well plates and a maximum concentration of 46.7 mM in 384-well plates. Since this concentration, in particular in the 96-well plates, is too low for optimum activity of the hyperpolarization-activated cation channels, different volumes are tested for the individual steps.

It has been found that the dye concentrations can be reduced to half of those in the protocol given by Molecular Devices.

In 96-well plates, good results are obtained even with the following volumes: 45 µl of wash buffer supernatant above the cells, 60 µl of dye in the wash buffer, 195 µl addition volume in the FLIPR. Such a high additional volume allows a maximum concentration of Na^+ + Cs^+ of 91 mM, i.e. at 8-10 mM CsCl, the final NaCl concentration can be 81-83 mM. For 80 mM Na^+ and 8 mM Cs^+, 6.43 µl of wash buffer, 171.43 µl of sodium buffer and 17.14 µl of cesium buffer are required, based on an added volume of 195 µl.

Materials and Methods:

The following materials and methods were, and can be, used to practice the invention as described in the Examples above. Other materials and methods can be used to practice other embodiments of the invention. Thus, the invention is not limited to the materials and methods disclosed below.

1. Solutions and buffers for the measurement with $DiBac_4(3)$

A: $DiBac_4(3)$ bis-(1,3-dibutylbarbituric acid)trimethine oxonol
From Molecular Probes, Cat. No. B-438, MW: 516.64 g/mol

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A 10 mM stock solution of $DiBac_4(3)$ is made up in DMSO (25 mg of $DiBac_4(3)$/4.838 ml of DMSO). Aliquots of this stock solution are stored at - 20°C.
Final concentration during dyeing and addition: 5 µM.

B: Forskolin MW: 410.5 g/mol
Final concentration during dyeing: 10 µM
Aliquots of a 10 mM stock solution in DMSO are stored at - 20°C.

C: Wash buffer: (140 mM choline chloride, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1 M KOH)

D: Presoak solution for saturating the tips of the pipettes:
as wash buffer + 10 µM $DiBac_4(3)$
This solution is only used for the presoak plate.

E: Dye solution: double concentrated, i.e. wash buffer + 10 µM $DiBac_4(3)$ + 20 µM forskolin

F: 10-fold concentrated solution for the addition plate: 500 mM NaCl in H_2O + 5 µM $DiBac_4(3)$
All substances are made up in this solution in 10-fold concentrated form.
Positive control (final concentration): 50 mM NaCl
Negative control (final concentration): 50 mM NaCl + 8 mM CsCl

2. Solutions and buffers for the measurements with the FLIPR Membrane Potential Assay Kit from Molecular Devices

A: FLIPR Membrane Potential Assay Kit,
from Molecular Probes, Cat. No. R8034

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B: Wash buffer: (140 mM choline chloride, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1M KOH).

C: Dye buffer: (content of one of the "reagent vials" of the FLIPR Membrane Potential Assay Kit in 10 ml of wash buffer)

D: Sodium buffer: (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1M KOH).

E: Cesium buffer: (140 mM CsCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1M KOH).

3. Cell culture operations:

The day before the measurement, the muHCN2-transfected CHO cells are seeded at a density of 35 000 cells/well, in each case in 200 µl of complete medium, into black 96-well microtiter plates. The cells are incubated at 37°C and 5% CO_2 overnight.

4. Dyeing with $DiBac_4(3)$ and measurement in FLIPR:

Before dyeing, the cells are washed three times with 400 µl of wash buffer in a cell washer. After the last washing step, a residual volume of 90 µl of wash buffer/well remains above the cells.

The washed cells (with 90 µl of wash buffer/well) are in each case incubated with 90 µl of dye solution/well at 37°C in the CO_2 incubator for 30 minutes. After this incubation time, the cell plate is measured in the FLIPR at about 37°C (preset temperature setting of the FLIPR manufacturer for measurement of membrane potentials with $DiBac_4(3)$), either immediately or after five minutes of thermostating.

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The snapshot (initial fluorescence before the start of the measurement) should on average be about 35 000 units. In the maximum, the FLIPR can resolve up to about 65 000 units.

When the program is started, the tips of the pipettes are initially saturated by immersion into presoak solution with $DiBac_4(3)$. Following this step, the actual measurement is initiated with the first measurement (t = 0 seconds). Since $DiBac_4(3)$ is a slow-response dye, it is sufficient to determine the fluorescence in the wells of the microtiter plate every 5 seconds. After about 20 seconds, the substances, which are present in the addition plate in 10-fold concentrated form, are added simultaneously to the microtiter plate using the pipettor. Since the volume after dyeing is 180 µl, 20 µl are added to each well. The measurement of the fluorescence can be terminated after about 5 minutes. For evaluation, the change in fluorescence in the interval where it is linear and in which uninhibited HCN2-transfected cells differ significantly from inhibited cells is examined.

5. Dyeing with the FLIPR Membrane Potential Assay Kit and measurement in the FLIPR.

Before dyeing, the cells are washed three times with 400 µl of wash buffer in a cell washer. After the last washing step, a residual volume of 45-90 µl of wash buffer/well remains above the cells.

Following addition of the dye solution (the volume depends on the desired final concentrations), the samples are incubated at room temperature (preferred) or at 37°C in a CO_2 incubator for 30 minutes. Following this incubation time, the cell plate is measured at room temperature in the FLIPR.

In the FLIPR Membrane Potential Assay Kit the snapshot (initial fluorescence before the start of the measurement) may be lower than that during the measurement with $DiBac_4(3)$, since the assay kit is more sensitive to changes in the membrane potential than $DiBac_4(3)$.

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Owing to the higher achievable sensitivity, the measurement should, wherever possible (FLIPRII), be carried out using an emission filter which is transparent to light above 550 nm. However, it is also possible to carry out the measurements using the standard filter, which is transparent between 510 and 580 nm.

When the program is started (t = 0), the FLIPR initially determines the fluorescence of all wells of the plate a number of times, before the depolarization is started after about 20 seconds by addition of sodium ions. In each case, the addition solution is mixed from the three buffers (wash buffer, sodium buffer and cesium buffer) such that the addition results in no change of the osmolarity, or in a change which is identical in all wells. The measurement of the fluorescence can be terminated after about 5 minutes. The wells to which, in addition to Na^+, 8 mM Cs^+ were added to block the HCN channel completely serve as negative control. By deducting these values from the others, a good measure for the activity of the HCN channel under the influence of the substance to be examined is obtained. For evaluation, the change in fluorescence in the interval where it is linear and in which uninhibited HCN2-transfected cells differ significantly from inhibited cells is examined.

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All references disclosed herein, including the following references, are hereby incorporated herein by reference.

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Table 1: SEQ ID NO. 1 Protein sequence of huHCN2

Accession number: AAC28444

```
   1 MDARGGGGRP GESPGASPTT GPPPPPPPRP PKQQPPPPPP PAPPPGPGPA PPQHPPRAEA
  61 LPPEAADEGG PRGRLRSRDS SCGRPGTPGA ASTAKGSPNG ECGRGEPQCS PAGPEGPARG
 121 PKVSFSCRGA ASGPAPGPGP AEEAGSEEAG PAGEPRGSQA SFMQRQFGAL LQPGVNKFSL
 181 RMFGSQKAVE REQERVKSAG AWIIHPYSDF RFYWDFTMLL FMVGNLIIIP VGITFFKDET
 241 TAPWIVFNVV SDTFFLMDLV LNFRTGIVIE DNTEIILDPE KIKKKYLRTW FVVDFVSSIP
 301 VDYIFLIVEK GIDSEVYKTA RALRIVRFTK ILSLLRLLRL SRLIRYIHQW EEIFHMTYDL
 361 ASAVMRICNL ISMMLLLCHW DGCLQFLVPM LQDFPRNCWV SINGMVNHSW SELYSFALFK
 421 AMSHMLCIGY GRQAPESMTD IWLTMLSMIV GATCYAMFIG HATALIQSLD SSRRQYQEKY
 481 KQVEQYMSFH KLPADFRQKI HDYYEHRYQG KMFDEDSILG ELNGPLREEI VNFNCRKLVA
 541 SMPLFANADP NFVTAMLTKL KFEVFQPGDY IIREGTIGKK MYFIQHGVVS VLTKGNKEMK
 601 LSDGSYFGEI CLLTRGRRTA SVRADTYCRL YSLSVDNFNE VLEEYPMMRR AFETVAIDRL
 661 DRIGKKNSIL LHKVQHDLNS GVFNNQENAI IQEIVKYDRE MVQQAELGQR VGLFPPPPPP
 721 PQVTSAIATL QQAAAMSFCP QVARPLVGPL ALGSPRLVRR PPPGPAPAAA SPGPPPPASP
 781 PGAPASPRAP RTSPYGGLPA APLAGPALPA RRLSRASRPL SASQPSLPHG APGPAASTRP
 841 ASSSTPRLGP TPAARAAAPS PDRRDSASPG AAGGLDPQDS ARSRLSSNL
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Table 2: SEQ ID NO. 2 Nucleotide sequence of huHCN2

Accession number: AF065164

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   1 CGGCTCCGCT CCGCACTGCC CGGCGCCGCC TCGCCATGGA CGCGCGCGGG GGCGGCGGGC
  61 GGCCCGGGGA GAGCCCGGGC GCGAGCCCCA CGACCGGGCC GCCGCCGCCG CCGCCCCCGC
 121 GCCCCCCCAA ACAGCAGCCG CCGCCGCCGC CGCCGCCCGC GCCCCCCCCG GGCCCCGGGC
 181 CCGCGCCCCC CCAGCACCCG CCCCGGGCCG AGGCGTTGCC CCCGGAGGCG GCGGATGAGG
 241 GCGGCCCGCG GGGCCGGCTC CGCAGCCGCG ACAGCTCGTG CGGCCGCCCC GGCACCCCGG
 301 GCGCGGCGAG CACGGCCAAG GGCAGCCCGA ACGGCGAGTG CGGGCGCGGC GAGCCGCAGT
 361 GCAGCCCCGC GGGGCCCGAG GGCCCGGCGC GGGGGCCCAA GGTGTCGTTC TCGTGCCGCG
 421 GGGCGGCCTC GGGGCCCGCG CCGGGGCCGG GGCCGGCGGA GGAGGCGGGC AGCGAGGAGG
 481 CGGGCCCGGC GGGGGAGCCG CGCGGCAGCC AGGCCAGCTT CATGCAGCGC CAGTTCGGCG
 541 CGCTCCTGCA GCCGGGCGTC AACAAGTTCT CGCTGCGGAT GTTCGGCAGC CAGAAGGCCG
 601 TGGAGCGCGA GCAGGAGCGC GTCAAGTCGG CGGGGGCCTG GATCATCCAC CCGTACAGCG
 661 ACTTCAGGTT CTACTGGGAC TTCACCATGC TGCTGTTCAT GGTGGGAAAC CTCATCATCA
 721 TCCCAGTGGG CATCACCTTC TTCAAGGATG AGACCACTGC CCCGTGGATC GTGTTCAACG
 781 TGGTCTCGGA CACCTTCTTC CTCATGGACC TGGTGTTGAA CTTCCGCACC GGCATTGTGA
 841 TCGAGGACAA CACGGAGATC ATCCTGGACC CCGAGAAGAT CAAGAAGAAG TATCTGCGCA
 901 CGTGGTTCGT GGTGGACTTC GTGTCCTCCA TCCCCGTGGA CTACATCTTC CTTATCGTGG
 961 AGAAGGGCAT TGACTCCGAG GTCTACAAGA CGGCACGCGC CCTGCGCATC GTGCGCTTCA
1021 CCAAGATCCT CAGCCTCCTG CGGCTGCTGC GCCTCTCACG CCTGATCCGC TACATCCATC
1081 AGTGGGAGGA GATCTTCCAC ATGACCTATG ACCTGGCCAG CGCGGTGATG AGGATCTGCA
1141 ATCTCATCAG CATGATGCTG CTGCTCTGCC ACTGGGACGG CTGCCTGCAG TTCCTGGTGC
1201 CTATGCTGCA GGACTTCCCG CGCAACTGCT GGGTGTCCAT CAATGGCATG GTGAACCACT
1261 CGTGGAGTGA ACTGTACTCC TTCGCACTCT TCAAGGCCAT GAGCCACATG CTGTGCATCG
1321 GGTACGGCCG GCAGGCGCCC GAGAGCATGA CGGACATCTG GCTGACCATG CTCAGCATGA
1381 TTGTGGGTGC CACCTGCTAC GCCATGTTCA TCGGCCACGC CACTGCCCTC ATCCAGTCGC
1441 TGGACTCCTC GCGGCGCCAG TACCAGGAGA AGTACAAGCA GGTGGAGCAG TACATGTCCT
1501 TCCACAAGCT GCCAGCTGAC TTCCGCCAGA AGATCCACGA CTACTATGAG CACCGTTACC
1561 AGGGCAAGAT GTTTGACGAG GACAGCATCC TGGGCGAGCT CAACGGGCCC CTGCGGGAGG
1621 AGATCGTCAA CTTCAACTGC CGGAAGCTGG TGGCCTCCAT GCCGCTGTTC GCCAACGCCG
1681 ACCCCAACTT CGTCACGGCC ATGCTGACCA AGCTCAAGTT CGAGGTCTTC CAGCCGGGTG
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1741 ACTACATCAT CCGCGAAGGC ACCATCGGGA AGAAGATGTA CTTCATCCAG CACGGCGTGG
1801 TCAGCGTGCT CACTAAGGGC AACAAGGAGA TGAAGCTGTC CGATGGCTCC TACTTCGGGG
1861 AGATCTGCCT GCTCACCCGG GGCCGCCGCA CGGCGAGCGT GCGGGCTGAC ACCTACTGCC
1921 GCCTCTATTC GCTGAGCGTG GACAACTTCA ACGAGGTGCT GGAGGAGTAC CCCATGATGC
1981 GGCGCGCCTT CGAGACGGTG GCCATCGACC GCCTGGACCG CATCGGCAAG AAGAATTCCA
2041 TCCTCCTGCA CAAGGTGCAG CATGACCTCA ACTCGGGCGT ATTCAACAAC CAGGAGAACG
2101 CCATCATCCA GGAGATCGTC AAGTACGACC GCGAGATGGT GCAGCAGGCC GAGCTGGGTC
2161 AGCGCGTGGG CCTCTTCCCG CCGCCGCCGC CGCCGCCGCA GGTCACCTCG GCCATCGCCA
2221 CGCTGCAGCA GGCGGCGGCC ATGAGCTTCT GCCCGCAGGT GGCGCGGCCG CTCGTGGGGC
2281 CGCTGGCGCT CGGCTCGCCG CGCCTCGTGC GCCGCCCGCC CCCGGGGCCC GCACCTGCCG
2341 CCGCCTCACC CGGGCCCCCG CCCCCCGCCA GCCCCCCGGG CGCGCCCGCC AGCCCCCGGG
2401 CACCGCGGAC CTCGCCCTAC GGCGGCCTGC CCGCCGCCCC CCTTGCTGGG CCCGCCCTGC
2461 CCGCGCGCCG CCTGAGCCGC GCGTCGCGCC CACTGTCCGC CTCGCAGCCC TCGCTGCCTC
2521 ACGGCGCCCC CGGCCCCGCG GCCTCCACAC GCCCGGCCAG CAGCTCCACA CCGCGCTTGG
2581 GGCCCACGCC CGCTGCCCGG GCCGCCGCGC CCAGCCCGGA CCGCAGGGAC TCGGCCTCAC
2641 CCGGCGCCGC CGGCGGCCTG GACCCCCAGG ACTCCGCGCG CTCGCGCCTC TCGTCCAACT
2701 TGTGACCCTC GCCGACCGCC CCGCGGGCCC AGGCGGGCCG GGGGCGGGGC CGTCATCCAG
2761 ACCAAAGCCA TGCCATTGCG CTGCCCCGGC CGCCAGTCCG CCCAGAAGCC ATAGACGAGA
2821 CGTAGGTAGC CGTAGTTGGA CGGACGGGCA GGGCCGGCGG GGCAGCCCCC TCCGCGCCCC
2881 CGGCCGTCCC CCCTCATCGC CCCGCGCCCA CCCCCATCGC CCCTGCCCCC GGCGGCGGCC
2941 TCGCGTGCGA GGGGGCTCCC TTCACCTCGG TGCCTCAGTT CCCCCAGCTG TAAGACAGGG
3001 ACGGGGCGGC CCAGTGGCTG AGAGGAGCCG GCTGTGGAGC CCCGCCCGCC CCCCACCCTC
3061 TAGGTGGCCC CCGTCCGAGG AGGATCGTTT TCTAAGTGCA ATACTTGGCC CGCCGGCTTC
3121 CCGCTGCCCC CATCGCGCTC ACGCAATAAC CGGCCCGGCC CCCGTCCGCG CGCGTCCCCC
3181 GGTGACCTCG GGGAGCAGCA CCCCGCCTCC CTCCAGCACT GGCACCGAGA GGCAGGCCTG
3241 GCTGCGCAGG GCGCGGGGGG GAGGCTGGGG TCCCGCCGCC GTGATGAATG TACTGACGAG
3301 CCGAGGCAGC AGTGCCCCCA CCGTGGCCCC CCACGCCCCA TTAACCCCCA CACCCCCATT
3361 CCGCGCAATA AA
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Table 3: SEQ ID NO. 3 Protein sequence of huHCN4

Accession number: HSA132429

```
   1  MDKLPPSMRK RLYSLPQQVG AKAWIMDEEE DAEEEGAGGR QDPSRRSIRL
  51  RPLPSPSPSA AAGGTESRSS ALGAADSEGP ARGAGKSSTN GDCRRFRGSL
 101  ASLGSRGGGS GGTGSGSSHG HLHDSAEERR LIAEGDASPG EDRTPPGLAA
 151  EPERPGASAQ PAASPPPPQQ PPQPASASCE QPSVDTAIKV EGGAAAGDQI
 201  LPEAEVRLGQ AGFMQRQFGA MLQPGVNKFS LRMFGSQKAV EREQERVKSA
 251  GFWIIHPYSD FRFYWDLTML LLMVGNLIII PVGITFFKDE NTTPWIVFNV
 301  VSDTFFLIDL VLNFRTGIVV EDNTEIILDP QRIKMKYLKS WFMVDFISSI
 351  PVDYIFLIVE TRIDSEVYKT ARALRIVRFT KILSLLRLLR LSRLIRYIHQ
 401  WEEIFHMTYD LASAVVRIVN LIGMMLLLCH WDGCLQFLVP MLQDFPDDCW
 451  VSINNMVNNS WGKQYSYALF KAMSHMLCIG YGRQAPVGMS DVWLTMLSMI
 501  VGATCYAMFI GHATALIQSL DSSRRQYQEK YKQVEQYMSF HKLPPDTRQR
 551  IHDYYEHRYQ GKMFDEESIL GELSEPLREE IINFNCRKLV ASMPLFANAD
 601  PNFVTSMLTK LRFEVFQPGD YIIREGTIGK KMYFIQHGVV SVLTKGNKET
 651  KLADGSYFGE ICLLTRGRRT ASVRADTYCR LYSLSVDNFN EVLEEYPMMR
 701  RAFETVALDR LDRIGKKNSI LLHKVQHDLN SGVFNYQENE IIQQIVQHDR
 751  EMAHCAHRVQ AAASATPTPT PVIWTPLIQA PLQAAAATTS VAIALTHHPR
 801  LPAAIFRPPP GSGLGNLGAG QTPRHLKRLQ SLIPSALGSA SPASSPSQVD
 851  TPSSSSFHIQ QLAGFSAPAG LSPLLPSSSS SPPPGACGSP SAPTPSAGVA
 901  ATTIAGFGHF HKALGGSLSS SDSPLLTPLQ PGARSPQAAQ PSPAPPGARG
 951  GLGLPEHFLP PPPSSRSPSS SPGQLGQPPG ELSLGLATGP LSTPETPPRQ
1001  PEPPSLVAGA SGGASPVGFT PRGGLSPPGH SPGPPRTFPS APPRASGSHG
1051  SLLLPPASSP PPPQVPQRRG TPPLTPGRLT QDLKLISASQ PALPQDGAQT
1101  LRRASPHSSG ESMAAFPLFP RAGGGSGGSG SSGGLGPPGR PYGAIPGQHV
1151  TLPRKTSSGS LPPPLSLFGA RATSSGGPPL TAGPQREPGA RPEPVRSKLP
1201  SNL*
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Table 4: SEQ ID NO. 4 Nucleotide sequence of huHCN4
Accession number: HSA132429

```
   1 GGTCGCTGGG CTCCGCTCGG TTGCGGCGGG AGCCCCGGGA CGGGCCGGAC GGGCCGGGGC
  61 AGAGGAGGCG AGGCGAGCTC GCGGGTGGCC AGCCACAAAG CCCGGGCGGC GAGACAGACG
 121 GACAGCCAGC CCTCCCGCGG GACGCACGCC CGGGACCCGC GCGGGCCGTG CGCTCTGCAC
 181 TCCGGAGCGG TTCCCTGAGC GCCGCGGCCG CAGAGCCTCT CCGGCCGGCG CCCATTGTTC
 241 CCCGCGGGGG CGGGGCGCCT GGAGCCGGGC GGCGCGCCGC GCCCCTGAAC GCCAGAGGGA
 301 GGGAGGGAGG CAAGAAGGGA GCGCGGGGTC CCCGCGCCCA GCCGGGCCCG GGAGGAGGTG
 361 TAGCGCGGCG AGCCCGGGGA CTCGGAGCGG GACTAGGATC CTCCCCGCGG CGCGCAGCCT
 421 GCCCAAGCAT GGGCGCCTGA GGCTGCCCCC ACGCCGGCGG CAAAGGACGC GTCCCCACGG
 481 GCGGACTGAC CGGCGGGCGG ACCTGGAGCC CGTCCGCGGC GCCGCGCTCC TGCCCCCGGC
 541 CCGGTCCGAC CCCGGCCCCT GGCGCCATGG ACAAGCTGCC GCCGTCCATG CGCAAGCGGC
 601 TCTACAGCCT CCCGCAGCAG GTGGGGGCCA AGGCGTGGAT CATGGACGAG GAAGAGGACG
 661 CCGAGGAGGA GGGGGCCGGG GGCCGCCAAG ACCCCAGCCG CAGGAGCATC CGGCTGCGGC
 721 CACTGCCCTC GCCCTCCCCC TCGGCGGCCG CGGGTGGCAC GGAGTCCCGG AGCTCGGCCC
 781 TCGGGGCAGC GGACAGCGAA GGGCCGGCCC GCGGCGCGGG CAAGTCCAGC ACGAACGGCG
 841 ACTGCAGGCG CTTCCGCGGG AGCCTGGCCT CGCTGGGCAG CCGGGGCGGC GGCAGCGGCG
 901 GCACGGGGAG CGGCAGCAGT CACGGACACC TGCATGACTC CGCGGAGGAG CGGCGGCTCA
 961 TCGCCGAGGG CGACGCGTCC CCCGGCGAGG ACAGGACGCC CCCAGGCCTG GCGGCCGAGC
1021 CCGAGCGCCC CGGCGCCTCG GCGCAGCCCG CAGCCTCGCC GCCGCCGCCC CAGCAGCCAC
1081 CGCAGCCGGC CTCCGCCTCC TGCGAGCAGC CCTCGGTGGA CACCGCTATC AAAGTGGAGG
1141 GAGGCGCGGC TGCCGGCGAC CAGATCCTCC CGGAGGCCGA GGTGCGCCTG GGCCAGGCCG
1201 GCTTCATGCA GCGCCAGTTC GGGGCCATGC TCCAACCCGG GGTCAACAAA TTCTCCCTAA
1261 GGATGTTCGG CAGCCAGAAA GCCGTGGAGC GCGAACAGGA GAGGGTCAAG TCGGCCGGAT
1321 TTTGGATTAT CCACCCCTAC AGTGACTTCA GATTTTACTG GGACCTGACC ATGCTGCTGC
1381 TGATGGTGGG AAACCTGATT ATCATTCCTG TGGGCATCAC CTTCTTCAAG GATGAGAACA
1441 CCACACCCTG GATTGTCTTC AATGTGGTGT CAGACACATT CTTCCTCATC GACTTGGTCC
1501 TCAACTTCCG CACAGGGATC GTGGTGGAGG ACAACACAGA GATCATCCTG GACCCGCAGC
1561 GGATTAAAAT GAAGTACCTG AAAAGCTGGT TCATGGTAGA TTTCATTTCC TCCATCCCCG
1621 TGGACTACAT CTTCCTCATT GTGGAGACAC GCATCGACTC GGAGGTCTAC AAGACTGCCC
1681 GGGCCCTGCG CATTGTCCGC TTCACGAAGA TCCTCAGCCT CTTACGCCTG TTACGCCTCT
1741 CCCGCCTCAT TCGATATATT CACCAGTGGG AAGAGATCTT CCACATGACC TACGACCTGG
1801 CCAGCGCCGT GGTGCGCATC GTGAACCTCA TCGGCATGAT GCTCCTGCTC TGCCACTGGG
1861 ACGGCTGCCT GCAGTTCCTG GTACCCATGC TACAGGACTT CCCTGACGAC TGCTGGGTGT
1921 CCATCAACAA CATGGTGAAC AACTCCTGGG GGAAGCAGTA CTCCTACGCG CTCTTCAAGG
1981 CCATGAGCCA CATGCTGTGC ATCGGCTACG GGCGGCAGGC GCCCGTGGGC ATGTCCGACG
2041 TCTGGCTCAC CATGCTCAGC ATGATCGTGG GTGCCACCTG CTACGCCATG TTCATTGGCC
2101 ACGCCACTGC CCTCATCCAG TCCCTGGACT CCTCCCGGCG CCAGTACCAG GAAAAGTACA
2161 AGCAGGTGGA GCAGTACATG TCCTTTCACA AGCTCCCGCC CGACACCCGG CAGCGCATCC
2221 ACGACTACTA CGAGCACCGC TACCAGGGCA AGATGTTCGA CGAGGAGAGC ATCCTGGGCG
2281 AGCTAAGCGA GCCCCTGCGG GAGGAGATCA TCAACTTTAA CTGTCGGAAG CTGGTGGCCT
2341 CCATGCCACT GTTTGCCAAT GCGGACCCCA ACTTCGTGAC GTCCATGCTG ACCAAGCTGC
2401 GTTTCGAGGT CTTCCAGCCT GGGGACTACA TCATCCGGGA AGGCACCATT GGCAAGAAGA
2461 TGTACTTCAT CCAGCATGGC GTGGTCAGCG TGCTCACCAA GGGCAACAAG GAGACCAAGC
2521 TGGCCGACGG CTCCTACTTT GGAGAGATCT GCCTGCTGAC CCGGGGCCGG CGCACAGCCA
2581 GCGTGAGGGC CGACACCTAC TGCCGCCTCT ACTCGCTGAG CGTGGACAAC TTCAATGAGG
2641 TGCTGGAGGA GTACCCCATG ATGCGAAGGG CCTTCGAGAC CGTGGCGCTG GACCGCCTGG
2701 ACCGCATTGG CAAGAAGAAC TCCATCCTCC TCCACAAAGT CCAGCACGAC CTCAACTCCG
2761 GCGTCTTCAA CTACCAGGAG AATGAGATCA TCCAGCAGAT TGTGCAGCAT GACCGGGAGA
2821 TGGCCCACTG CGCGCACCGC GTCCAGGCTG CTGCCTCTGC CACCCCAACC CCCACGCCCG
2881 TCATCTGGAC CCCGCTGATC CAGGCACCAC TGCAGGCTGC CGCTGCCACC ACTTCTGTGG
2941 CCATAGCCCT CACCCACCAC CCTCGCCTGC CTGCTGCCAT CTTCCGCCCT CCCCCAGGAT
3001 CTGGGCTGGG CAACCTCGGT GCCGGGCAGA CGCCAAGGCA CCTGAAACGG CTGCAGTCCC
3061 TGATCCCTTC TGCGCTGGGC TCCGCCTCGC CCGCCAGCAG CCCGTCCCAG GTGGACACAC
3121 CGTCTTCATC CTCCTTCCAC ATCCAACAGC TGGCTGGATT CTCTGCCCCC GCTGGACTGA
3181 GCCCACTCCT GCCCTCATCC AGCTCCTCCC CACCCCCCGG GGCCTGTGGC TCCGCCTCGG
3241 CTCCCACACC ATCAGCTGGC GTAGCCGCCA CCACCATAGC CGGGTTTGGC CACTTCCACA
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3301 AGGCGCTGGG TGGCTCCCTG TCCTCCTCCG ACTCTCCCCT GCTCACCCCG CTGCAGCCAG
3361 GCGCCCGCTC CCCGCAGGCT GCCCAGCCAT CTCCCGCGCC ACCCGGGGCC CGGGGAGGCC
3421 TGGGACTCCC GGAGCACTTC CTGCCACCCC CACCCTCATC CAGATCCCCG TCATCTAGCC
3481 CCGGGCAGCT GGGCCAGCCT CCCGGGGAGT TGTCCCTAGG TCTGGCCACT GGCCCACTGA
3541 GCACGCCAGA GACACCCCCA CGGCAGCCTG AGCCGCCGTC CCTTGTGGCA GGGGCCTCTG
3601 GGGGGGCTTC CCCTGTAGGC TTTACTCCCC GAGGAGGTCT CAGCCCCCCT GGCCACAGCC
3661 CAGGCCCCCC AAGAACCTTC CCGAGTGCCC CGCCCCGGGC CTCTGGCTCC CACGGATCCT
3721 TGCTCCTGCC ACCTGCATCC AGCCCCCCAC CACCCCAGGT CCCCCAGCGC CGGGGCACAC
3781 CCCCGCTCAC CCCCGGCCGC CTCACCCAGG ACCTCAAGCT CATCTCCGCG TCTCAGCCAG
3841 CCCTGCCTCA GGACGGGGCG CAGACTCTCC GCAGAGCCTC CCCGCACTCC TCAGGGGAGT
3901 CCATGGCTGC CTTCCCGCTC TTCCCCAGGG CTGGGGGTGG CAGCGGGGGC AGTGGGAGCA
3961 GCGGGGGCCT CGGTCCCCCT GGGAGGCCCT ATGGTGCCAT CCCCGGCCAG CACGTCACTC
4021 TGCCTCGGAA GACATCCTCA GGTTCTTTGC CACCCCCTCT GTCTTTGTTT GGGGCAAGAG
4081 CCACCTCTTC TGGGGGGCCC CCTCTGACTG CTGGACCCCA GAGGGAACCT GGGGCCAGGC
4141 CTGAGCCAGT GCGCTCCAAA CTGCCATCCA ATCTATGAGC TGGGCCCTTC CTTCCCTCTT
4201 CTTTCTTCTT TTCTCTCCCT TCCTTCTTCC TTCAGGTTTA ACTGTGATTA GGAGATATAC
4261 CAATAACAGT AATAATTATT TAAAAAACCA CACACACCAG AAAAACAAAA GACAGCAGAA
4321 AATAACCAGG TATTCTTAGA GCTATAGATT TTTGGTCACT TGCTTTTATA GACTATTTTA
4381 ATACTCAGCA CTAGAGGGAG GGAGGGGGAG GGAGGAGGGA GCAGGCAGGT CCCAAATGCA
4441 AAAGCCAGAG AAAGGCAGAT GGGGTCTCCG GGGCTGGGCA GGGGTGGGAG TGGCCAGTGT
4501 TGGCGGTTCT TAGAGCAGAT GTGTCATTGT GTTCATTTAG AGAAACAGCT GCCATCAGCC
4561 CGTTAGCTGT AACTTGGAGC TCCACTCTGC CCCCAGAAAG GGGCTGCCCT GGGGTGTGCC
4621 CTGGGGAGCC TCAGAAGCCT GCGACCTTGG GAGAAAAGGG CCAGGGCCCT GAGGGCCTAG
4681 CATTTTTTCT ACTGTAAACG TAGCAAGATC TGTATATGAA TATGTATATG TATATGTATG
4741 TAAGATGTGT ATATGTATAG CTATGTAGCG CTCTGTAGAG CCATGTAGAT AGCCACTCAC
4801 ATGTGCGCAC ACGTGTGCGG TCTAGTTTAA TCCCATGTTG ACAGGATGCC CAGGTCACCT
4861 TACACCCAGC AACCCGCCTT GGCCCGCAGG CTGTGCACTG CATGGTCTAG GGACGTTCTC
4921 TCTCCAGTCC TCAGGGAAGA GGACGCCAGG ACTTCGCAGC AGGCCCCCTC TCTCCCCATC
4981 TCTGGTCTCA AAGCCAGTCC CAGCCTGACC TCTCACCACA CGGAAGTGGA AGACTCCCCT
5041 TTCCTAGGGC CTCAAGCACA CACCG
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Table 5: SEQ ID NO. 5 Protein sequence of muHCN2

Accession number: CAA12406

```
  1 MDARGGGGRP GDSPGTTPAP GPPPPPPPPA PPQPQPPPAP PPNPTTPSHP ESADEPGPRA
 61 RLCSRDSACT PGAAKGGANG ECGRGEPQCS PEGPARGPKV SFSCRGAASG PSAAEEAGSE
121 EAGPAGEPRG SQASFLQRQF GALLQPGVNK FSLRMFGSQK AVEREQERVK SAGAWIIHPY
181 SDFRFYWDFT MLLFMVGNLI IIPVGITFFK DETTAPWIVF NVVSDTFFLM DLVLNFRTGI
241 VIEDNTEIIL DPEKIKKKYL RTWFVVDFVS SIPVDYIFLI VEKGIDSEVY KTARALRIVR
301 FTKILSLLRL LRLSRLIRYI HQWEEIFHMT YDLASAVMRI CNLISMMLLL CHWDGCLQFL
361 VPMLQDFPSD CWVSINNMVN HSWSELYSFA LFKAMSHMLC IGYGRQAPES MTDIWLTMLS
421 MIVGATCYAM FIGHATALIQ SLDSSRRQYQ EKYKQVEQYM SFHKLPADFR QKIHDYYEHR
481 YQGKMFDEDS ILGELNGPLR EEIVNFNCRK LVASMPLFAN ADPNFVTAML TKLKFEVFQP
541 GDYIIREGTI GKKMYFIQHG VVSVLTKGNK EMKLSDGSYF GEICLLTRGR RTASVRADTY
601 CRLYSLSVDN FNEVLEEYPM MRRAFETVAI DRLDRIGKKN SILLHKVQHD LSSGVFNNQE
661 NAIIQEIVKY DREMVQQAEL GQRVGLFPPP PPPQVTSAIA TLQQAVAMSF CPQVARPLVG
721 PLALGSPRLV RRAPPGPLPP AASPGPPAAS PPAAPSSPRA PRTSPYGVPG SPATRVGPAL
781 PARRLSRASR PLSASQPSLP HGVPAPSPAA SARPASSSTP RLGPAPTART AAPSPDRRDS
841 ASPGAASGLD PLDSARSRLS SNL
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Table 6: SEQ ID NO. 6 Nucleotide sequence of muHCN2

Accession number: MMJ225122

```
   1 CCGCTCCGCT CCGCACTGCC CGGCGCCGCC TCGCCATGGA TGCGCGCGGG GGCGGCGGGC
  61 GGCCGGGCGA TAGTCCGGGC ACGACCCCTG CGCCGGGGCC GCCGCCACCG CCGCCGCCGC
 121 CCGCGCCCCC TCAGCCTCAG CCACCACCCG CGCCACCCCC GAACCCCACG ACCCCCTCGC
 181 ACCCGGAGTC GGCGGACGAG CCCGGCCCGC GCGCCCGGCT CTGCAGCCGC GACAGCGCCT
 241 GCACCCCTGG CGCGGCCAAG GGCGGCGCGA ATGGCGAGTG CGGGCGCGGG GAGCCGCAGT
 301 GCAGCCCCGA GGGCCCCGCG CGCGGCCCCA AGGTTTCGTT CTCATGCCGC GGGGCGGCCT
 361 CCGGGCCCTC GGCGGCCGAG GAGGCGGGCA GCGAGGAGGC GGGCCCGGCG GGTGAGCCGC
 421 GCGGCAGCCA GGCTAGCTTC CTGCAGCGCC AATTCGGGGC GCTTCTGCAG CCCGGCGTCA
 481 ACAAGTTCTC CCTGCGGATG TTCGGCAGCC AGAAGGCCGT GGAGCGCGAG CAGGAACGCG
 541 TGAAGTCGGC GGGGGCCTGG ATCATCCACC CCTACAGCGA CTTCAGGTTC TACTGGGACT
 601 TCACCATGCT GTTGTTCATG GTGGGAAATC TCATTATCAT TCCCGTGGGC ATCACTTTCT
 661 TCAAGGACGA GACCACCGCG CCCTGGATCG TCTTCAACGT GGTCTCGGAC ACTTTCTTCC
 721 TCATGGACTT GGTGTTGAAC TTCCGCACCG GCATTGTTAT TGAGGACAAC ACGGAGATCA
 781 TCCTGGACCC CGAGAAGATA AAGAAGAAGT ACTTGCGTAC GTGGTTCGTG GTGGACTTCG
 841 TGTCATCCAT CCCGGTGGAC TACATCTTCC TCATAGTGGA GAAGGGAATC GACTCCGAGG
 901 TCTACAAGAC AGCGCGTGCT CTGCGCATCG TGCGCTTCAC CAAGATCCTC AGTCTGCTGC
 961 GGCTGCTGCG GCTATCACGG CTCATCCGAT ATATCCACCA GTGGGAAGAG ATTTTCCACA
1021 TGACCTACGA CCTGGCAAGT GCAGTGATGC GCATCTGTAA CCTGATCAGC ATGATGCTAC
1081 TGCTCTGCCA CTGGGACGGT TGCCTGCAGT TCCTGGTGCC CATGCTGCAA GACTTCCCCA
1141 GCGACTGCTG GGTGTCCATC AACAACATGG TGAACCACTC GTGGAGCGAG CTCTACTCGT
1201 TCGCGCTCTT CAAGGCCATG AGCCACATGC TGTGCATCGG CTACGGGCGG CAGGCGCCCG
1261 AGAGCATGAC AGACATCTGG CTGACCATGC TCAGCATGAT CGTAGGCGCC ACCTGCTATG
1321 CCATGTTCAT TGGGCACGCC ACTGCGCTCA TCCAGTCCCT GGATTCGTCA CGGCGCCAAT
1381 ACCAGGAGAA GTACAAGCAA GTAGAGCAAT ACATGTCCTT CCACAAACTG CCCGCTGACT
1441 TCCGCCAGAA GATCCACGAT TACTATGAAC ACCGGTACCA AGGGAAGATG TTTGATGAGG
1501 ACAGCATCCT TGGGGAACTC AACGGGCCAC TGCGTGAGGA GATTGTGAAC TTCAACTGCC
1561 GGAAGCTGGT GGCTTCCATG CCGCTGTTTG CCAATGCAGA CCCCAACTTC GTCACAGCCA
1621 TGCTGACAAA GCTCAAATTT GAGGTCTTCC AGCCTGGAGA TTACATCATC CGAGAGGGGA
1681 CCATCGGGAA GAAGATGTAC TTCATCCAGC ATGGGGTGGT GAGCGTGCTC ACCAAGGGCA
1741 ACAAGGAGAT GAAGCTGTCG GATGGCTCCT ATTTCGGGGA GATCTGCTTG CTCACGAGGG
1801 GCCGGCGTAC GGCCAGCGTG CGAGCTGACA CCTACTGTCG CCTCTACTCA CTGAGTGTGG
1861 ACAATTTCAA CGAGGTGCTG GAGGAATACC CCATGATGCG GCGTGCCTTT GAGACTGTGG
1921 CTATTGACCG GCTAGATCGC ATAGGCAAGA AGAACTCCAT CTTGCTGCAC AAGGTTCAGC
1981 ATGATCTCAG CTCAGGTGTG TTCAACAACC AGGAGAATGC CATCATCCAG GAGATTGTCA
2041 AATATGACCG TGAGATGGTG CAGCAGGCAG AGCTTGGCCA GCGTGTGGGG CTCTTCCCAC
2101 CACCGCCACC ACCGCAGGTC ACATCGGCCA TTGCCACCCT ACAGCAGGCT GTGGCCATGA
2161 GCTTCTGCCC GCAGGTGGCC CGCCCGCTCG TGGGGCCCCT GGCGCTAGGC TCCCCACGCC
2221 TAGTGCGCCG CGCGCCCCCA GGGCCTCTGC CTCCTGCAGC CTCGCCAGGG CCACCCGCAG
2281 CAAGCCCCCC GGCTGCACCC TCGAGCCCTC GGGCACCGCG GACCTCACCC TACGGTGTGC
2341 CTGGCTCTCC GGCAACGCGC GTGGGGCCCG CATTGCCCGC ACGTCGCCTG AGCCGCGCCT
2401 CGCGCCCACT GTCCGCCTCG CAGCCCTCGC TGCCCCATGG CGTGCCCGCG CCCAGCCCCG
2461 CGGCCTCTGC GCGCCCGGCC AGCAGCTCCA CGCCGCGCCT GGGACCCGCA CCCACCGCCC
2521 GGACCGCCGC GCCCAGTCCG GACCGCAGGG ACTCAGCCTC GCCGGGCGCT GCCAGTGGCC
2581 TCGACCCACT GGACTCTGCG CGCTCGCGCC TCTCTTCCAA CTTGTGACCC TTGAGCGCCG
2641 CCCCGCGGGC CGGGCGGGGC CGTCATCCAC ACCAAAGCCA TGCCTCGCGC CGCCCGCCCG
2701 TGCCCGTGCA GAAGCCATAG AGGGACGTAG GTAGCTTAGG AGGCGGGCGG CCCTGCGCCC
2761 GGCTGTCCCC CCATCGCCCT GCGCCCACCC CCATCGCCCC TGCCCCAGCG GCGGCCGCAC
2821 GGGAGAGGGA GGGGTGCGAT CACCTCGGTG CCTCAGCCCC AACCTGGGAC AGGGACAGGG
2881 CGGCCCTGGC CGAGGACCTG GCTGTGCCCC GCATGTGCGG TGGCCTCCGA GGAAGAATAT
2941 GGATCAAGTG CAATACACGG CCAAGCCGGC GTGGGGGTGA GGCTGGGTCC CCGGCCGTCG
3001 CCATGAATGT ACTGACGAGC CGAGGCAGCA GTGGCCCCCA CGCCCCATTA ACCCACAACC
3061 CCATTCCGCG CAATAAACGA CAGCATTGGC AAAAAAAAAA AA
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Table 7: Abbreviations

AKT	Arabidopsis thaliana K+ transport
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
EDTA	ethylenediamine tetraacetic acid
FLIPR	fluorescence imaging plate reader
HAC	hyperpolarization-activated cation channel; this name was used by some groups
HCN	hyperpolarization-activated cyclic nucleotide gated cation channel; this is the new, generally accepted term
HEK	human embryonic kidney;
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HTS	high-thoughput screening
KAT	K+ channel from Arabidopsis thaliana